

A High Fat/High Carbohydrate Diet Induces Aortic Valve Disease in C57BL/6J Mice

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OBJECTIVES	The purpose of this study was to compare aortic valve function and morphology in adult wild-type (WT) mice and in low-density lipoprotein receptor-deficient (LDLr ^{-/-}) mice fed or not fed a high-fat/high-carbohydrate (HF/HC) diet.
BACKGROUND	Observations suggest a link between degenerative aortic valve stenosis (AS) and atherosclerosis. Aortic valve stenosis has been successfully induced in animal models of extreme hypercholesterolemia, but these models are less relevant to humans. It is not known if a proatherogenic HF/HC diet without added cholesterol could have the same negative impacts.
METHODS	Forty C57BL/6J mice were divided into four groups: WT + normal diet, WT + HF/HC diet, LDLr ^{-/-} with a normal diet, and LDLr ^{-/-} with a HF/HC diet. Aortic valve function and histology were evaluated by echocardiography after four months.
RESULTS	Wild-type mice on a HF/HC diet became mildly hypercholesterolemic, obese, and hyperglycemic. As expected, LDLr ^{-/-} mice became severely hypercholesterolemic. Both WT and LDLr ^{-/-} mice on a HF/HC diet displayed smaller valve areas and higher transvalvular velocities ($p < 0.01$) after four months. Aortic valve leaflets were thicker and infiltrated with lipids and macrophages in both HF/HC groups.
CONCLUSIONS	A HF/HC diet in mice results in significant aortic valve abnormalities. Putting WT mice on a HF/HC diet reproduced a combination of atherogenic factors (obesity, mild dyslipidemia, and hyperglycemia) more commonly encountered in humans than isolated severe hypercholesterolemia. Severe hypercholesterolemia was not a prerequisite in our model. This experimental model suggests that AS development is multifactorial and that hypercholesterolemia should not be the only target in this disease. (J Am Coll Cardiol 2006;47:850-5) © 2006 by the American College of Cardiology Foundation

Degenerative calcific aortic valve stenosis (AS) is the most common valvular heart disease potentially leading to valve replacement in North America. Recent observations have suggested a link between AS and atherosclerosis. Many traditional atherogenic factors, such as hypercholesterolemia, obesity, and diabetes, have been associated with a higher prevalence of AS (1-3). Therefore, many investigators now focus on the hypothesis of an atherosclerosis-AS link. Hypercholesterolemia has been recurrently incriminated as a risk factor for developing AS. Cholesterol-lowering drugs (statins) are currently under investigation in prospective trials evaluating their potential to alter AS progression (4-7).

Investigators have tried to reproduce AS in animal models of atherosclerosis. We and others have reported significant aortic valve abnormalities in a classic experimental atherosclerosis model (cholesterol-fed New Zealand rabbit) (8-11). This model of extremely severe hypercholesterolemia leads to characteristic atherosclerosis-like lesions in the aortic valve leaflets. This type of animal model remains very useful but such high levels of blood cholesterol

are rarely if ever encountered in humans. Considering that AS has been statistically associated with milder metabolic abnormalities in human retrospective studies, we hypothesized that a less aggressive atherogenic diet, without high cholesterol contents, could significantly alter the aortic valve. The current study was therefore designed to assess if a high-fat/high-carbohydrate (HF/HC) diet (12) without added cholesterol can alter aortic valve function and morphology in mice.

METHODS

Animals. C57BL/6J mice, which are genetically prone to develop diet-induced obesity and atherosclerotic lesions, were chosen for this protocol. Forty adult male (20 to 25 g), wild type (WT) or low-density lipoprotein receptor deficient (LDLr^{-/-} (C57BL/6J background) (Jackson Laboratories, Bar Harbor, Massachusetts), were divided into four groups (10 animals/group): group 1: WT + normal diet (used as normal controls); group 2: WT + HF/HC diet; group 3: LDLr^{-/-} + normal diet; and group 4: LDLr^{-/-} + HF/HC diet. All animals were followed for four months. This protocol was made in accordance with the recommendations of the Canadian Council for Animal Care and was approved by the local animal protection committee.

Diets. Normal diet consisted of normal mouse chow (Mouse Colony Chow #5018; Purina Mills, St. Louis,

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Abbreviations and Acronyms

AS	= aortic valve stenosis
AVA	= aortic valve area
HF/HC	= high fat/high carbohydrate
LDLr ^{-/-}	= low-density lipoprotein receptor-deficient
WT	= wild type

Missouri). The HF/HC diet was purchased from BioServ (F3282; Frenchtown, New Jersey). In this HF/HC diet, 59% of total calories are provided from fats (high palmitic acid content) without added cholesterol. Both diets were available to the animals without restriction.

Data collection. At the end of protocol, mice were fasted overnight, anesthetized with ketamine/xylazine, had an echocardiogram (see the following section), and were thereafter killed by exsanguination. Heart, lungs, livers and abdominal fat were rapidly collected, weighed, and snap frozen in liquid nitrogen for further analysis. Blood samples were taken for the measurement of fasting total cholesterol, glucose, and insulin levels. Plasma levels of TNF- α and interleukin-6 were also evaluated using specific mouse enzymatic immunoassays (Assay Designs, Ann Arbor, Michigan).

Echocardiography. A two-dimensional and Doppler echocardiogram was performed at baseline and two months and just before death at four months under ketamine/xylazine anesthesia. Ultrasound images were obtained with a 12-MHz phased-array probe connected to a Sonos 5500 echograph (Philips Medical Imaging, Andover, Massachusetts). Aortic valve area (AVA) was measured by continuity equation. Parasternal long axis view was used to measure the diameter of the left ventricular outflow tract as well as to observe the morphology and opening of the aortic leaflets.

Histology. At death, a portion of the ascending aorta including the aortic valve was dissected, rinsed in phosphate-buffered saline, fixed in paraformaldehyde, and mounted in paraffin. Cross-sectional serial sections were prepared and von Kossa's silver stain used to visualize calcium deposits.

Aortic valve leaflet thickness. Good-quality leaflet sections of each group ($n = 5/\text{group}$) were selected, and leaflet thickness (middle portion) was measured using an image analysis software (SigmaScan, Systat Software, Point Richmond, California) by an investigator blinded to the treatment group. Normal leaflets (WT + normal diet [group 1])

were used as controls and the leaflet thickness of this group was arbitrarily set at 100%.

Immunohistochemistry. The Vectastain system (Vector Laboratories, Burlingame, California) was used for immunostaining. Monoclonal mouse anti-CD68 antibodies (NeoMarkers, Fremont, California) were used, followed by a secondary peroxidase-conjugated rabbit antimouse antibody for the identification of macrophage cells. Labeling for osteopontin (Clone 1B20, Assay Designs) and T-lymphocytes (anti-CD3, clone UCHT1; Dako Diagnostics Inc., Mississauga, Ontario, Canada) were performed similarly.

Statistics. Results are presented as mean \pm SEM unless specified otherwise. Repeated measures one-way analysis of variance with post-hoc Tukey test was used for intragroup comparisons over time, and standard one-way analysis with post-hoc Tukey test was used for intergroup comparison. Unpaired t test was used for comparisons between two groups. Statistical significance was set at $p < 0.05$.

RESULTS

Animal characteristics (Table 1). The LDLr^{-/-} mice on a normal diet did not gain any weight during the protocol and therefore remained significantly smaller than all the other groups. Their heart, lung, and liver weights behaved accordingly and remained smaller.

All animals on the HF/HC diet (both WT and LDLr^{-/-}) were significantly overweight compared to WT + normal diet. At death, total heart weight as well as liver weight was significantly increased in both WT and LDLr^{-/-} on a HF/HC diet. Intra-abdominal fat was increased by a mean of 60% in WT + HF/HC diet compared to WT + normal diet ($p < 0.01$).

Fasting cholesterol, glucose, and insulin levels (Table 2). Compared to WT + normal diet, cholesterol levels were mildly elevated in WT + HF/HC diet to levels comparable to LDLr^{-/-} + normal diet and extremely elevated in LDLr^{-/-} + HF/HC diet ($p < 0.01$ vs. all other groups). The LDL cholesterol levels were increased by the HF/HC diet in normal animals, but these levels remained relatively low. As expected, LDL levels in LDLr^{-/-} animals were higher than in WT animals.

Fasting glucose and insulin levels remained similar to controls in the WT animals fed with the HF/HC diet. Insulin levels tended to be slightly lower in this HF/HC

Table 1. Animal Characteristics After Four Months on Normal or HF/HC Diet

	WT		LDLr ^{-/-}	
	Normal	HF/HC	Normal	HF/HC
Body weight, g	41.2 \pm 1.2	55.9 \pm 2.0*	28.2 \pm 0.6†	50.8 \pm 2.3*
Heart weight, mg	148 \pm 2	182 \pm 8*	122 \pm 2†	166 \pm 5*
Lung weight, mg	190 \pm 13	188 \pm 14	147 \pm 3†	175 \pm 6
Liver weight, mg	1,575 \pm 84	2,533 \pm 209*	1,062 \pm 47†	1,866 \pm 192*

Results are expressed as mean \pm SEM. Statistical analysis: one-way ANOVA with post-hoc Tukey test. * $p < 0.05$ vs. corresponding group on normal diet; † $p < 0.05$ vs. WT on normal diet.

WT = wild type; LDLr^{-/-} = low-density lipoprotein-deficient mice; HF/HC = high-fat/high-carbohydrate diet.

Table 2. Serum Cholesterol, LDL, Glucose, and Insulin Levels After Four Months

	WT		LDLr ^{-/-}	
	Normal	HF/HC	Normal	HF/HC
Total cholesterol, mmol/l (mg/dl)	2.52 ± 0.23 (97)	4.30 ± 0.41 (166)*	6.37 ± 0.26 (246)*	18.74 ± 2.26 (722)*†
LDL-cholesterol, mmol/l (mg/dl)	0.15 ± 0.012 (5.8)	1.18 ± 0.089 (45.5)*	3.17 ± 0.359 (122.5)*	9.15 ± 1.531 (353.1)*†
Glucose, mmol/l (mg/dl)	14.4 ± 1.0 (260)	14.9 ± 1.7 (268)	23.6 ± 1.3 (425)*	28.6 ± 1.0 (515)*†
Insulin, pmol/l (μIU/ml)	212 ± 35 (31)	166 ± 40 (24)	15 ± 2 (2)*	48 ± 14 (7)*†
Glucose/insulin ratio	0.09 ± 0.021	0.16 ± 0.055	1.88 ± 0.327*	0.91 ± 0.178*

Data are presented as mean ± SEM (n = 10). Statistical analysis: one-way ANOVA with post-hoc Tukey test. *p < 0.05 vs. WT + normal diet; †p < 0.05 vs. LDLr^{-/-} + normal diet.

Abbreviations as in Table 1.

group (p = NS). Fasting blood glucose levels were significantly higher than normal reported values for fasting glucose in normal mice in both WT groups. Insulin levels were within normal reported values. Both LDLr^{-/-} groups had very high blood glucose levels combined with severely decreased insulin levels compatible with overt diabetes mellitus with decreased insulin production.

Mediators of inflammation. Inflammatory mediators interleukin-6 and TNF-alpha were measured and remained low, without any significant difference between normal and HF/HC diets in WT mice.

Echocardiographic assessment. Aortic valve leaflets were more echogenic after four months in all groups compared with WT + normal diet. Aortic valve area was significantly smaller in all groups compared with WT + normal diet. Considering the large differences in body weight between groups, valve area was indexed for body weight as shown in Table 3. Once corrected for body weight, valve area in LDLr^{-/-} with normal diet group was not reduced and remained comparable to WT + normal diet. Only animals on a HF/HC diet, WT and LDLr^{-/-}, had smaller valve areas despite correction for body weight. Similar results were found for maximal transvalvular velocities. Only animals on the HF/HC diet displayed increased transvalvular velocities. Figures 1A and 1B shows the parallel evolution of AVA and body weight over time during the four-month protocol in WT animals. In the WT + normal diet, both body weight and AVA increased during the course of the protocol, whereas the HF/HC animals gained more weight and their AVA remained unchanged. Increases in transvalvular velocities became significant after two to four months on the HF/HC diet (Fig. 1C). Typical examples of trans-

valvular velocities in WT on normal (top panel) and HF/HC (bottom panel) diets are shown in Figure 2.

Leaflet thickness (Fig. 3). Leaflet thickness was significantly increased in WT mice fed with the HF/HC diet compared with the WT + normal diet. A typical example of leaflet thickening in the WT + HF/HC diet group is illustrated in Figure 4. Similar observations were made in LDLr^{-/-} + HF/HC group (not shown).

Immunohistochemistry (Fig. 4). Macrophage infiltration (CD68-positive cells) and foam cells were clearly evident in the thickened valve leaflets of the animals in groups fed with the HF/HC diet. Osteopontin expression and T-lymphocyte infiltrates were also detected (not shown) as well as some nodular calcium deposition by von Kossa's stain.

DISCUSSION

We report for the first time in an animal model that aortic valve disease can be initiated by a high-fat/high-carbohydrate diet. Contrary to previously published animal models of the disease which were exclusively associated with severe hypercholesterolemia, we demonstrate in this study that a HF/HC diet with a low cholesterol content not only results in mild dyslipidemia, obesity, and hyperglycemia in these mice but also induces significant aortic valve abnormalities both in vivo (smaller valve area and higher transvalvular velocities) and ex vivo (leaflet thickening, lipid and macrophage infiltrates, signs of calcification). The abnormalities found in the valves of the animals closely resemble those found in the early phases of AS (13,14).

Numerous epidemiologic and histologic studies have suggested a link between proatherogenic factors and AS

Table 3. Echocardiographic Evaluation of Aortic Valve in Vivo After Four Months of Normal or HF/HC Diet

	WT		LDLr ^{-/-}	
	Normal	HF/HC	Normal	HF/HC
AVA (mm ²)	1.62 ± 0.07	1.22 ± 0.07*	1.02 ± 0.05*	1.16 ± 0.06*
AVAi (mm ² /mg)	39.3 ± 1.2	21.8 ± 0.9*	36.2 ± 1.6	24.0 ± 1.6*†
Max vel (cm/s)	83.6 ± 1.5	108.1 ± 7.4*	79.2 ± 2.1	99.8 ± 3.7*†

Results are expressed as mean ± SEM. Statistical analysis: one-way ANOVA with post-hoc Tukey test. *p < 0.05 vs. WT + normal diet; †p < 0.05 vs. LDLr^{-/-} + normal diet.

AVA = aortic valve area; AVAi = AVA indexed for body weight; Max Vel = maximal transvalvular velocities by continuous-wave Doppler; other abbreviations as in Table 1.

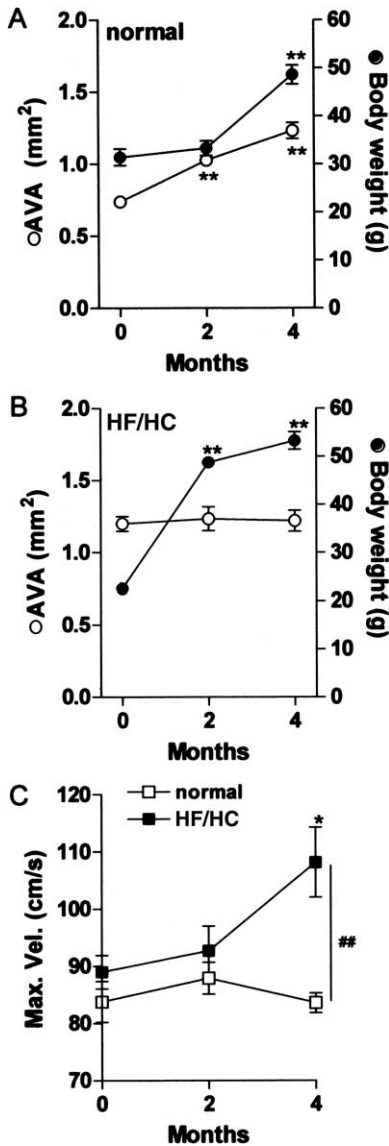


Figure 1. Aortic valve area (AVA) (open circles) and body weight (closed circles) evolution during the study in wild-type mice fed with a normal diet (A) or a high-fat/high-carbohydrate (HF/HC) diet (B). (C) Maximal transvalvular velocities (cm/s) over time during the study. Results are presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$ vs. baseline (month 0) by repeated measures analysis of variance with Tukey post-hoc test. ## $p < 0.01$ between indicated values by unpaired t test.

(1,15,16). High LDL, low high-density lipoprotein, high lipoprotein(a), cigarette smoking, obesity, diabetes mellitus, hypertension, and high C-reactive protein have all been shown to be statistically associated with AS (3,5,6,17–22). Hypercholesterolemia has been recurrently associated with AS in epidemiologic and histologic studies. Low-density lipoprotein deposits in the diseased leaflets have been clearly demonstrated (23). In order to better understand the pathophysiology of the disease and its link with hypercholesterolemia, animal models of aortic valve sclerosis/stenosis have been developed (9–11,24). It has clearly been shown in these models of extreme severe hypercholesterolemia that aortic leaflet lesions sharing similarities with those found in

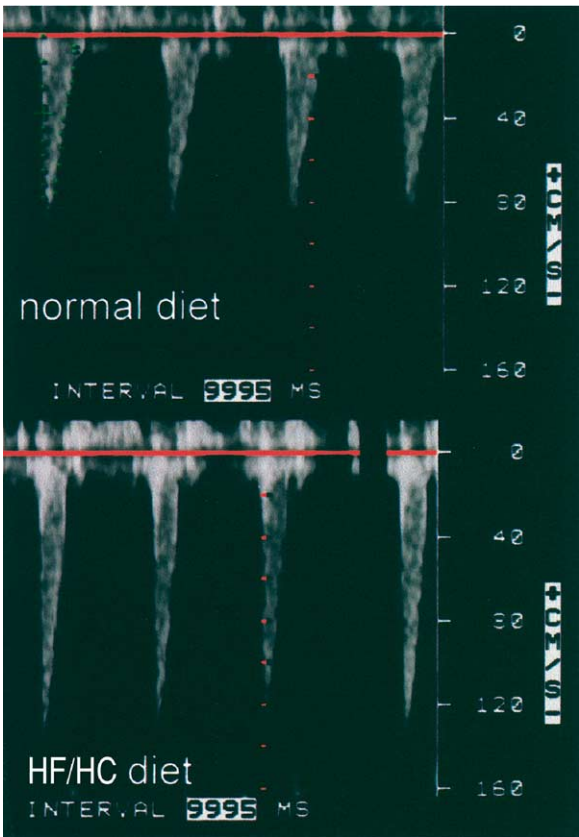


Figure 2. Typical examples of maximal transvalvular aortic velocities in wild-type mice fed with normal diet (top) or high-fat/high-carbohydrate (HF/HC) diet (bottom) by continuous-wave Doppler.

humans can be reproduced (9–11). However the development of AS in severely hypercholesterolemic animals is not reminiscent of the human disease, because such high levels of cholesterol are rarely (if ever) encountered in humans. These animal models remain very useful research tools but are clearly imperfect.

Treatment of hypercholesterolemia to slow the progression of AS remains controversial. Despite a clear statistical association in retrospective studies, a recently published

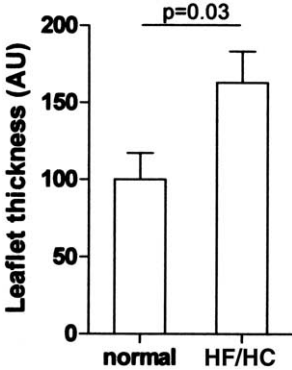


Figure 3. Leaflet thickness in wild-type mice fed with high-fat/high-carbohydrate (HF/HC) diet vs. normal diet. Results are expressed in arbitrary units (AU) with normal leaflet thickness set at 100. An unpaired t test was used for the statistical comparison.

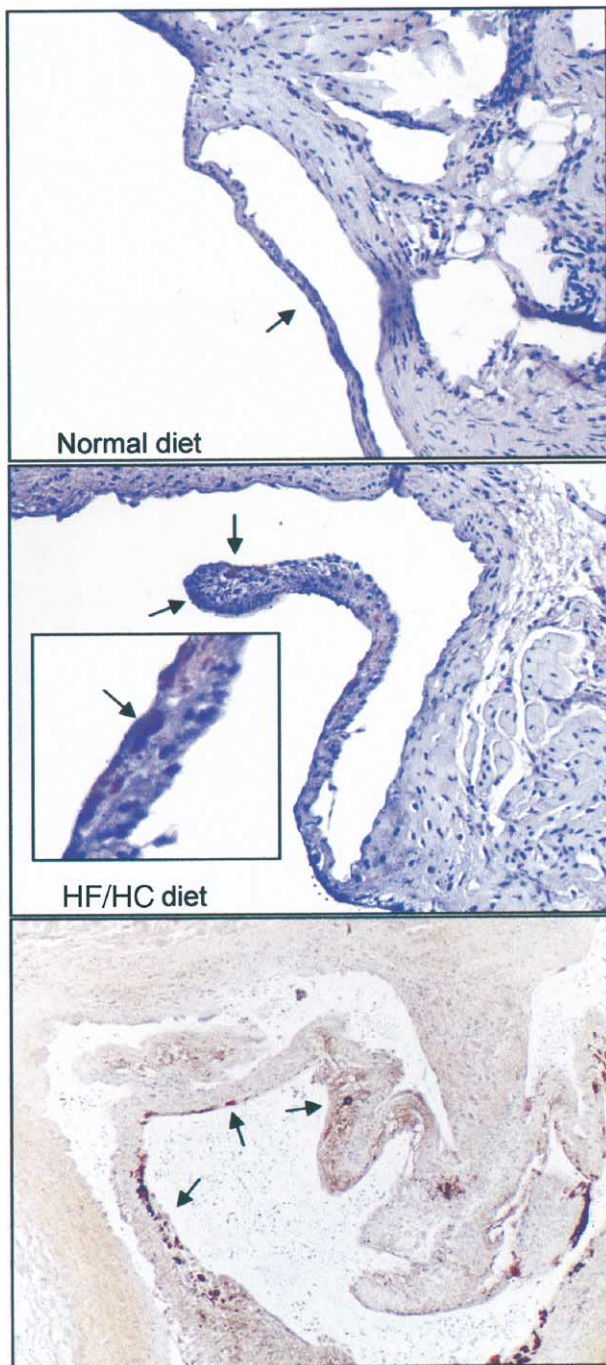


Figure 4. Increased aortic valve leaflet (arrows) thickness in wild-type mouse fed a high-fat/high-carbohydrate (HF/HC) diet (middle) compared with normal diet (top). Leaflets in HF/HC animals show positive labeling for macrophages (CD68-positive cells) and foam cells (arrows). Inset (middle section) shows a magnified view of the diseased leaflet. **Bottom panel** shows calcium deposits (arrow) in an aortic valve leaflet of an HF/HC diet-fed wild-type mouse by von Kossa's stain.

prospective trial using a statin has yielded negative results (25). Aortic valve stenosis is clearly a frequent disease in North America, and proatherogenic factors that have been statistically linked with AS are closely related to the North American lifestyle. Hypercholesterolemia is only one of

these factors. For these reasons, we evaluated if exposure to a “North American diet” consisting of high amounts of noncholesterol fats and carbohydrates could induce significant abnormalities in the aortic valve. In our WT animals, this diet induced abdominal obesity, mild hypercholesterolemia, and hyperglycemia. This is more reminiscent of the classic atherogenic factors found in North America. This combination of metabolic abnormalities in our animals also shares striking similarities with the human metabolic syndrome (26).

Considering that known animal models of the disease were related to severe hypercholesterolemia we also wanted to assess if this was a prerequisite for the development of aortic valve lesions. We therefore compared WT mice with mutants deficient in LDL receptors. Our results show that aortic valve abnormalities are found in both groups on the HF/HC diet even though the two groups had very different LDL cholesterol levels. Despite this striking difference in cholesterol levels, animals in both groups had decreased AVA and similar increases in transvalvular velocities after four months on the HF/HC diet. These results suggest that severe hypercholesterolemia is not a prerequisite to induce aortic valve disease in those mice. It also suggests that a combination of milder hypercholesterolemia with other atherogenic risk factors such as abdominal obesity and hyperglycemia may be enough to initiate the process. The worst combination of atherogenic factors capable of inducing the most severe and progressive aortic valve abnormalities in this animal model remains to be determined. However, our results clearly point toward a multifactorial etiology.

Study limitations. The current study was short-term (four months). It is possible that LDLr^{−/−} mice on normal diet had mild valve abnormalities that would have become more evident after a longer follow-up. The speed of progression of the disease was not assessed and we do not know if the animals with the most atherogenic factors would have progressed faster toward a more severe aortic valve disease. Blood pressure was not measured. Some animals might have developed some degree of hypertension, which has been previously linked to AS.

Conclusions. We report for the first time the development of aortic valve disease in obese, mildly hypercholesterolemic, hyperglycemic mice resulting from exposure to a high-fat/high-carbohydrate diet without added cholesterol. We have reproduced the early stage of aortic valve disease in this animal model in association with a mix of atherogenic risk factors more frequently encountered in humans in North America and sharing similarities with the human metabolic syndrome.

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